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Study on plasmatic metabolomics of Uygur patients with essential hypertension based on nuclear magnetic resonance technique

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Abstract. – OBJECTIVE: Metabolomics is the analysis of the global constitution of endogenous metabolites in cells, tissue, and bodily fluids based on analysis techniques with high output, high sensitivity, and high resolution. The physiological and pathological state of the subject investigated could be identified and analyzed through examining metabolite changes. In this study, 1H-NMR metabolomics was employed to study plasma metabolites of both Uygur patients with hypertension and healthy people, thereby filtering out characteristic metabolites for Uygur patients with hypertension. The pathogenesis of hypertension was discussed via metabolic pathways.

PATIENTS AND METHODS: A total of 256 Uygur subjects were recruited for this study and divided into two groups, namely hypertension group (157 Uygur patients with hypertension) and normal group (99 healthy Uygur subjects). They were all taken from epidemiological surveys on the Uygur people of Qira County, Hotan, and Xinjiang between 2009 and 2012 conducted by Hypertension Research Group of Xinjiang Medical University. Furthermore, all subjects have Uygur blood within three generations. For the hypertension group, the recruitment criteria is systolic blood pressure (SBP) ≥ 140 mmHg (lmmHg = 0.133 kPa) and/or diastolic blood pressure (DBP) ≥ 90 mmHg at rest. Patients who had taken antihypertensive drugs within two weeks and those who were diagnosed with essential hypertension (EH) were also included in this group, while patients with secondary hypertension, myocardiosis, congenital heart disease, and rheumatic valvular heart disease were not included. In the healthy normal-pressure group, blood pressures were within normal range: SBP < 140 mmHg, DBP < 90 mmHg, without history of antihypertensive drugs, cardiovascular and cerebrovascular diseases, and liver/kidney diseases. All subjects were measured separately with Inova600 nuclear magnetic resonance (NMR) spectrometer to conduct the 1H-NMR experiment. Serum specimens from both the hypertension group and the healthy control group were used for NMR spectrograms before data pre-processing, where aggregate analysis was performed for NMR data/metabolic information with principal component analysis (PCA). Then, partial least squares discriminant analysis (PLS-DA) was employed to classify and predict different groups of specimens, and orthogonal partial least squares discriminant analysis (OPLS-DA) was conducted to cross-validate the quality of the models. Statistical analysis was further performed to test significance of the correlation coefficient to determine differential metabolic components in serums of both groups of subjects. Based on the information from the differential metabolic components, a metabolic pathway network related to hypertension could be constructed, thereby revealing potential biomarkers for hypertension.

RESULTS: Clinical data showed that subjects in the two groups were not significantly different with respect to age, weight, and height, as well as lipid indices, including TG, LDL ($p > 0.05$), while FPG, SBP, DBP, HDL, and TC were significantly different between the two groups ($p < 0.05$). OPLS-DA results demonstrated that integral quantities of principal components were mainly distributed within four areas of the ellipse scatter diagram (95% confidence interval). From the score plot and 3D distribution diagram, it can be observed that the distribution areas for the two groups are completely separate, thereby indicating that the serum of the Uygur hypertension patients is significantly different from that of healthy subjects in terms of metabolic components. OPLS-DA results indicate that differences in metabolic components are significant between the two groups, and 12 different metabolites were identified. Compared to healthy subjects, patients with hypertension possess a much lower quantity of many amino acids, including valine, alanine, pyroracemic acid, inose, p-hydroxyphenylalanine, and methylhistidine, among others ($p < 0.05$), with a significant increase in VLDL, LDL, lactic acid, and acetone ($p < 0.05$).

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CONCLUSIONS: The 1H-NMR metabolomics process, in combination with OPLS-DA pattern identification, is an effective way to differentiate the serum metabolites characteristic of hypertension patients. Pattern identification analysis of NMR spectrum data with OPLS-DA could identify metabolites of hypertension patients versus healthy subjects. The metabolic phenotype of Uygur hypertension patients shows significant heteromorphosis, with the 12 characteristic metabolites as potential biomarkers of hypertension.

Key Words:
Essential hypertension, Insulin resistance, Uygur patients, Nuclear magnetic resonance tecnique.

Introduction

Essential hypertension (EH) is a clinical syndrome which manifests as increased systemic arterial pressure. Long-term hypertension can lead to severe damage to organs such as the heart, brain, and kidney. EH is always accompanied by a metabolic disorder, such as insulin resistance, hyperinsulinemia, abnormal lipid profile, and obesity. Based on analysis techniques with high output, high sensitivity, and high precision, metabolomics qualitatively and quantitatively examines the endogenous metabolites in bodily fluid and analyzes the different metabolic fingerprints of organisms in various conditions. Combined with chemo-informatic techniques like pattern recognition, it is possible to obtain their respective bio-markers and follow their metabolic pathways. The procedures in metabolomics are as follows: specimen preparation, metabolite separation, detection and identification, data analysis, and model establishment[1], involving technical means such as NMR[2,3], liquid chromatography-mass spectrometry (LC-MS)[4-6], gas chromatography-mass spectrometry[7-8], chromatogram such as HPLC[9,10], GC[11-13], and CE. Among them, 1H-NMR allows for non-invasive, non-partial detection of the specimen, therefore increasing authenticity and replicability. Since pathological changes in hypertension patients will result in metabolite changes accordingly, performing metabolomic analysis of these disease-induced metabolites not only facilitates our better understanding of both pathological processes and metabolic pathways of biomolecules to discover biomarkers, but assists in clinical diagnosis and provides targeted therapy and early prevention as well. Previous studies have shown that the incidence of EH varies significantly between regions and nations. Uygur is a minority nationality that has a high incidence of EH. However, presently there are few studies on metabolomics of Uygur patients with hypertension. Therefore, the objective of this study is to investigate metabolic changes and the biological basis of EH patients using NMR technique, to analyze the mechanism of metabolic changes, and to identify specific metabolic biomarkers.

Patients and Methods

Patients

A total of 256 Uygur subjects were recruited for this study and divided into two groups, namely the hypertension group (157 Uygur patients with hypertension) and the normal/control group (99 healthy Uygur subjects). They were all taken from epidemiological surveys on the Uygur people of Qira County, Hotan, and Xinjiang between 2009 and 2012 conducted by Hypertension Research Group of Xinjiang Medical University. Of note, each subject has Uygur blood within three generations. This study was approved by the Ethics Committee of Xinjiang Medical University, and prior to the investigation, all subjects signed an informed consent form with understanding of this study.

Diagnostic Criteria

Diagnostic criteria for the hypertension group is as follows (according to "Guidelines for the Prevention and Treatment of Hypertension in China" established by the revision committee in 2004): systolic blood pressure (SBP) ≥ 140 mmHg (lmmHg = 0.133 kPa) and/or diastolic blood pressure (DBP) ≥ 90 mmHg at rest. Patients who had taken antihypertensive drugs within 2 weeks and those who were diagnosed with EH were also included in this group, while patients with secondary hypertension, myocardiosis, congenital heart disease, and rheumatic valvular heart disease were not included.

Criteria for the healthy normal-pressure group: normal blood pressure (SBP < 140 mmHg, DBP < 90 mmHg), without history of antihypertensive drugs, cardiovascular and cerebrovascular diseases, and obvious liver/kidney diseases.

Inclusion Criteria

1. Age between 30 and 70. Hypertension patients should meet the above criteria while normal subjects had no family history of hypertension or antihypertensive drugs.

2. Subjects were collected from Uygur population of Hotan, Xinjiang between 2009 and 2012.
3. All subjects had no blood ties with one another and no history of miscegenation.
4. All subjects signed an informed consent form.

Exclusion Criteria

1. EH acute disease and uncertain EH diagnosis.
2. Subjects with cardiovascular diseases, such as diabetes mellitus, coronary disease, cerebral apoplexy, atherosclerosis, and cardiac insufficiency.
3. Subjects with liver, kidney, thyroid gland and hematological system diseases and trauma.
4. Pregnant and lactating females.

Investigation and Screening

General Information

Name, gender, age, disease progression, medical history, medication condition.

Clinical Indices

Height (m), weight (kg), blood pressure (SBP/DBP), fasting plasma glucose (FPG), total cholesterol (TC), triglycerides (TG), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C).

The procedures are as follows: (1) measuring height (m), weight (kg), and blood pressure (mmHg); (2) lipid and blood sugar: all subjects fasted for at least 12 hours after dinner of the day before physical examination. Venous blood is taken in the morning and anticoagulated with heparin sodium, centrifuged at 3,500 r/min, preparation of plasma, then kept at –80°C. The sample was further examined by the clinical laboratory of the First Affiliated Hospital of Xinjiang Medical University. FPG, TG, TC, LDL-C, and HDL-C were determined.

Blood Sample Collection

All subjects were required to fast for at least 12 hours before 5 mL of peripheral venous blood was collected with an EDTA-Na2 anticoagulated, vacuum-sealed blood collection tube. Plasma was separated by centrifuge, then filled into 5 mL test tubes and stored in –80°C conditions.

1H-NMR Data Collection

All specimens were examined separately with Inova600 nuclear magnetic resonance (NMR) spectrometer in NMR Laboratory of Analyzing and Investigation Center of Xinjiang Medical University. 1D NOESY pulse sequences with pre-saturation was adopted, using the following parameters: spectral width 8000 Hz, mixing time 0.15 seconds, relaxation delay time 2 seconds, number of sampling point 32 k, cumulative frequency 64. During relaxation delay and mixing time, the peak of water was suppressed by pre-saturation, saturation time of 2 seconds, spectral width of 10000 Hz, number of sampling point 32768 k, scanning time of 128, time for each scan 1.638 seconds, experiment temperature 300 K. All 1H-NMR spectrograms were obtained with the same spectrometer. Specimens of high quality, one-dimensional hydrogen spectrum were chosen for 2D NMR to identify the components, including total correlation spectroscopy (TOCSY), correlation spectroscopy (COSY), and J-Resolved spectroscopy (J-RES).

Data Pre-processing

Original NMR spectrograms frequently contain a large amount of data, high correlation of data points, and solvent peaks and thus cannot be directly used for multivariate statistical analysis. Pre-processing of NMR data is usually necessary. Phase modulation and baseline correction are key procedures in pre-processing, where Fourier transformation is performed for NMR data to obtain spectrograms and Topspin 2.0 is employed for manual baseline and phase modulation. Afterwards, in order to reduce the differences in spectrograms so that spectrograms obtained in various field strengths were comparable, and to eliminate chemical shift deviations induced by varying pH values, automatic piecewise-integration was conducted in areas of 1H = 9 ppm-0.5 ppm, with the integrating range at 0.003 ppm. Meanwhile, spectrum peak intensity of 1H chemical shift-ranging between 4.69-5.20 was set to 0 and eliminated from the data matrix to minimize the influence of residual water signals on analysis results. A 20 × 298 data matrix was derived through piecewise integration with each column representing one specimen. Then the matrix was saved as a text file and imported into Microsoft Excel.

Pattern Recognition Analysis

The two-dimensional matrix was imported into an Excel file with SIMCA-P+11 (Umetrics, Inc., Umea, Sweden) for multivariate pattern recognition analysis [14-16]. Then PLS-DA was

performed to distinguish differences in serum metabolites of the two groups. Determination of differential metabolic components should adopt correlation coefficients of metabolites obtained by OPLS-DA. Analysis results were presented as score blot and spatial distribution diagrams.

Statistical Analysis

Taking component indices of subjects in both groups as study factors, SPSS 13.0 was employed to process general data and for data analysis (SPSS Inc., Chicago, IL, USA). The measured data was presented as mean ± deviation (x±s), and t-test of two independent samples was conducted for inter-group comparison. $p < 0.05$ was considered statistically significant.

Results

Comparison of Clinical Data

Clinical data shows that subjects in the two groups are not significantly different with respect to age, weight, and height, as well as lipid indices, such as TG and LDL ($p > 0.05$), while the FPG, SBP, DBP, HDL, and TC were significantly different between the two groups ($p < 0.05$). It can be observed that Uygur patients with EH suffered from abnormal metabolites of blood pressure, blood sugar, and lipids, as shown in Table I.

OPLS-DA Score plot and Spatial Distribution Diagram of Serum 1H-NMR Spectrums for EH Group and Control Group

In this study, prediction parameters of the OPLS-DA analysis model were R2X = 0.11346, R2Y = 0.538279, and Q2 = 0.88. OPLS-DA results demonstrated that integral quantities of principal components mainly distribute into four areas of the ellipse scatter diagram (95% confidence interval). Based on the score plot and 3D distribution diagram, it can be seen that the distribution areas for the two groups were completely separate, therefore, confirming that plasma of the Uygur hypertension patients were significantly different than that of healthy subjects in terms of metabolic components ($p > 0.05$). The two groups had very little intersection and overlap, as illustrated in Figures 1 and 2.

OPLS-DA Analysis of Primary Differential Metabolites for EH Group and Control Group

OPLS-DA results indicated that differences in metabolic components were significant between the two groups, and 12 different metabolites were identified. Metabolites with positive correlation coefficients were lower in the plasma of the EH group than in the control group, whereas those with negative correlation coefficients were higher in the plasma of the EH group. Compared with healthy subjects, patients with hypertension demonstrated a greater reduction in many amino acids, including valine, alanine, pyroracemic acid, inose, p-hydroxyphenylalanine, and methylhistidine, among others. ($p < 0.05$), and a significant increase in VLDL, LDL, lactic acid, and acetone ($p < 0.05$), as shown in Table II.

Discussion

1H-NMR metabolomics can accomplish tests under physiological conditions or near-physiological conditions at certain temperatures and buffering ranges, and dynamically monitor and

Table I. Comparison of clinical data of Uygur EH patient group and healthy control group ($\bar{x} \pm s$).

Index	Control group	Hypertension group	p
Age (year)	54.46 ± 5.24	57.05 ± 1.76	0.56
Height (cm)	165.23 ± 9.98	162.70 ± 9.16	0.89
Weight (kg)	68.56 ±8.35	66.67 ± 8.13	0.25
DBP (mmHg)	128 ± 7.85	156 ± 8.24	0.00
SBP (mmHg)	86 ± 5.40	102 ± 6.27	0.00
FPG (mmol/l)	4.56 ± 0.56	11.60 ± 0.85	0.03
TC (mmol/l)	4.40 ± 0.11	4.97 ± 0.10	0.00
TG (mmol/l)	2.76 ± 0.08	5.40 ± 0.04	0.17
HDL (mmol/l)	1.43 ± 0.05	1.08 ± 0.01	0.01
LDL (mmol/l)	2.78 ± 0.02	2.94 ± 0.01	0.26

$p < 0.05$, significant difference.

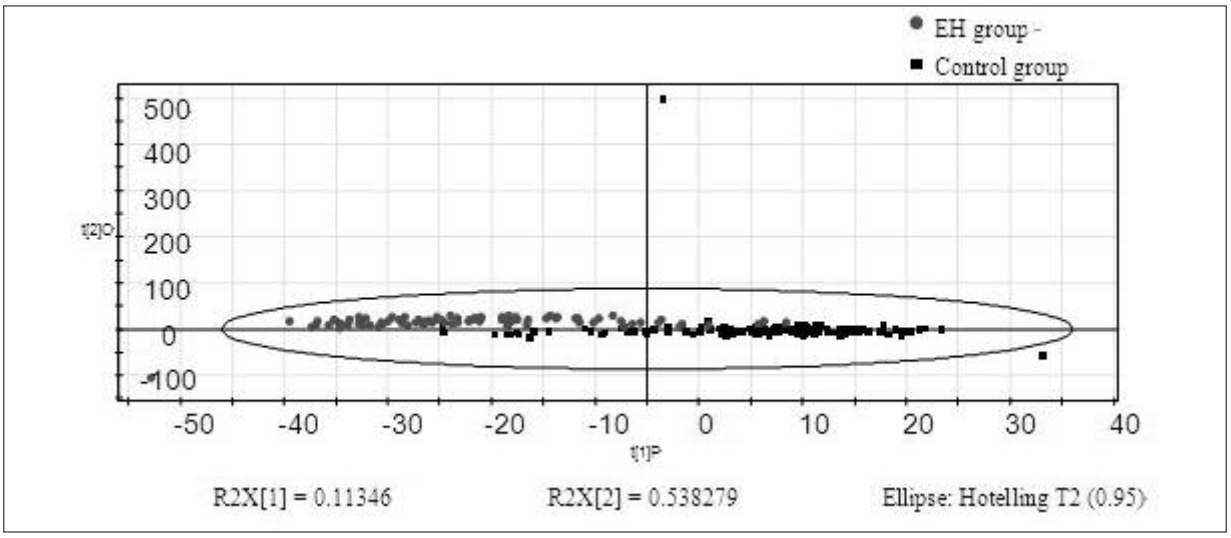

Figure 1. OPLS-DA score plot of serum 1H-NMR spectrums (CPMG pulse sequences) for EH group and control group.

quantitatively analyze differential metabolites induced by organic pathological changes. Through comparing and analyzing different expressions of molecular metabolites of the EH group and control group, and combining the metabolic data with biological events in pathophysiological processes, the variations of endogenous metabolites could be comprehensively and immediately recapitulated. Recent studies have proposed that hypertension is not merely limited to abnormal hemodynamics, which is incidentally a metabolic disease, but also coexists with metabolic disorders of lipid and carbohydrates. 1H-NMR metabolomics is applied to investigate the mechanism of hypertension, its fundamental basis for normal cell destruction and dysfunction, changes in the steady state of endogenous metabolites during carbohydrate metabolism, lipid metabolism, protein metabolism, tricarboxylic acid cycle and creatine synthesis, thereby altering bodily fluid components of patients directly or indirectly.

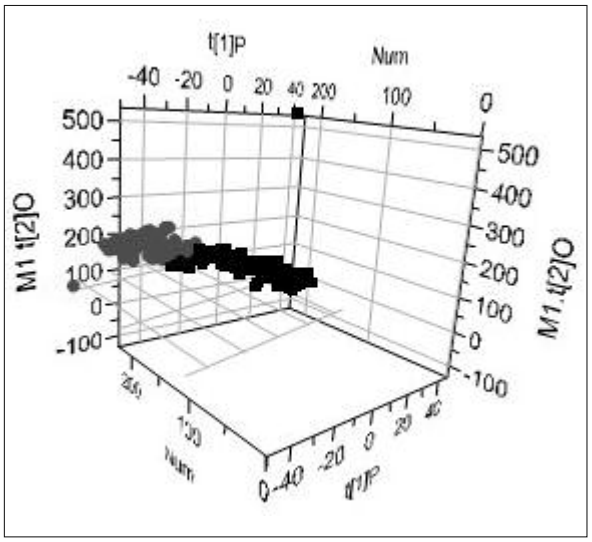

Figure 2. OPLS-DA spatial distribution diagram of plasma 1H-NMR spectrums (CPMG pulse sequences) for EH group and control group.

In 1987, Ferrannini et al[17] firstly firmed hypertensive patients have insulin resistance documented by the euglycemic insulin clamp technique. In order to confirm that hypertension coexists with metabolic diseases such as abnormal glucose metabolism and abnormal lipid profiles, and that some specific metabolites change accordingly, we constructed an OPLS model for the EH group and control group. OPLS score plot showed that sample points of the two groups were completely separate in the two-dimensional space, indicating significant differences in serum metabolic spectrum. Based on 1H-NMR metabolomics, Brindle et al[18] studied serum metabolic spectrums of 17 patients with SBP ≥ 150 mmHg, 19 patients with SBP from 131 to 149 mmHg and 28 patients with SBP ≤ 130 mmHg to investigate the relationship between serum metabolic spectrum and EH. The results showed that serum metabolic spectrums of patients with SBP ≤ 130 mmHg were significantly different from those with SBP ≥ 131 mmHg. Fur-

Table II. Differential metabolites and their correlation coefficients from OPLS-DA of plasma 1H-NMR spectrums for EH group and control group.

No.	Metabolites	Chemical shift/(mg·L-1)	Adscription	Correlation coefficient
1	VLDL	0.86(m)	CH3(CH2)n, CH3CH2CH2C	-0.70
2	Valine	0.99(d), 1.03(d), 3.60(d)	CH3, CH3, α-CH2	0.85
3	LDL	1.26(m)	CH3CH2(CH2)n, CH2OCOR	-0.81
4	Lactic acid	1.32(d), 4.11(q)	CH3, CH	-0.46
5	Alanine	1.47(d)	CH3, α-CH	0.31
6	Acetone	2.22(s)	CH3	-0.69
7	Acetylformic acid	2.39(s)	CH3	-0.63
8	β-glucose	3.24(dd), 3.41(t), 3.48(ddd), 3.91(dd)	C-H2, C-H4, C-H5, C-H3, C-H6, half CH2- CH6, C-H1	0.59
9	α- glucose	3.54(dd), 3.73(dd), 5.23(d)	C-H2, half CH2-CH6, C-H1	0.24
10	Inose	3.65(dd)	H4/H6	0.58
11	p-hydroxyphenylalanine	6.89(d), 7.1(d)	α-CH, H3/H5, H2/H6	0.43
12	Methylhistidine	7.05(s), 7.77(s)	H4	0.34

Notes: Is single peak, d is double peak, t is triple peak, q is quadruple peak, m is multiple peak, dd is double double peak, ddd is double double double peak. In intra-group comparison, metabolites with positive correlation coefficients are lower in EH group than control group, whereas those with negative correlation coefficients are higher in EH group. Results: Compared to control group, EH group demonstrates a greater reduction in valine, alanine, pyroracemic acid, inose, p-hydroxyphenylalanine, and methylhistidine, and a significant increase in VLDL, LDL, lactic acid, and acetone.

ther research demonstrated that the differences were due to varying lipid metabolism. On the other hand, plasma metabolic spectrums of patients with SBP ≥ 150 mm Hg and SBP ≥ 131 mmHg are similar and cannot be separated, indicating that variations in serum metabolic spectrums are somehow related to SBP. The results of this study indicated that metabolomics is different between EH group and control group, which sufficiently proved that the development of hypertension has metabolomic roots. Therefore, metabolomics could timely and accurately characterize the overall functional status of hypertension patients.

In Vivo Lipid Metabolic Pathway of Hypertension Patients

Regarding lipid metabolism, 1H-NMR pattern recognition analysis demonstrated increased VLDL and LDL in hypertension patients. Numerous research studies have demonstrated that many hypertension patients suffer from abnormal metabolism. For instance, cholesterol and triglyceride levels were much higher in hypertension patients than that of the healthy population, while HDL-C levels were lower. In addition, clinical research shows that among the risk factors for cardiovascular disease, abnormal lipids is somehow related to blood pressure[19,20]. Vascular dysfunction could be observed in patients with abnormal blood lipid levels or hypertension, rendering it an important mediator in the mutual influence between abnormal blood lipid level and hypertension[21]. The results of the current study showed that hypertension causes abnormal lipid metabolism, increased lipid levels, and reduced clearance, possibly relating to inflammation and decreased activity of lipoprotein lipase.

Energy Metabolic Pathway of Hypertension Patients

Regarding energy metabolism, the results showed that glucose content in the plasma of hypertension patients was significantly lower than that of the healthy population, different from clinical biochemical indices. The amount of pyruvate and lactate was significantly increased for hypertension patients. The changes in relative concentrations of glucose and pyruvate could reflect cell activity, oxygenation level (hypoxia, regional hypoxia, oxidative stress), pH value, and homeostasis[22]. According to the two pathways in glucose catabolism, gluconeogenesis and glycogenolysis, glucose is metabolized to pyruvate through glycogenolysis in aerobic conditions, while pyruvate produces glucose through gluconeogenesis in anaerobic conditions. For hypertension patients, glycogenolysis increases, while glycogen synthesis decreases, resulting in lowered glucose. Gluconeogenesis inhibition results in high production and low consumption of pyruvate and citrate, thereby, leading to their accumulation. Large-

scale oversea investigations found that 68.5% of hypertension patients had abnormal glycometabolism (diabetes mellitus, impaired fasting glucose, and abnormal sugar tolerance), and 65% of patients had two or more uncontrolled cardiovascular risk factors[23], indicating that diabetes mellitus usually coexisted with hypertension, therefore, having higher risk[24].

In Vivo Protein Metabolic Pathway in Hypertension Patients

Regarding protein metabolism, results indicated that compared to the control group, the EH group demonstrated a greater reduction in valine, alanine, pyroracemic acid, inose, p-hydroxyphenylalanine, and methylhistidine, among others. This results in a reduction of intermediates (citrate, alpha-ketoglutaric acid, succinyl-CoA, and fumarate) in the tri-carboxylic acid cycle, thereby interrupting protein synthesis and affecting functions such as genetic transcription, cell division, and autoimmune response. Thus, hypertension patients suffer from depleted protein stores, abnormal immunologic function, and slower healing ability. The content of amino acids in the plasma of hypertension patients is significantly different from healthy subjects, indicating abnormal amino acid metabolism. It could be hypothesized that the differing amino acid composition may be a potential bio-marker for hypertension.

Conclusions

NMR spectroscopy detected chemical compounds in bodily fluid from different metabolic pathways. The variation in these metabolites facilitates the detection of pathological changes. Through pattern recognition analysis, 1H-NMR metabolomics was used, revealing that serum metabolomics is significantly different between hypertension patients and healthy subjects. Specifically, differences in 12 metabolites were found to be statistically significant and may be potential biomarkers in hypertension patients, which could be adopted as a strong evaluation tool for predicting incidence and development of hypertension and its pathogenesis.

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Conflict of Interest

The Authors declare that there are no conflicts of interest.

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